Mechanical loading stimulates BMP7, but not BMP2, production by osteocytes

Santos, A.; Bakker, A.D.; Willems, H.M.E.; Bravenboer, N.; Bronckers, A.L.J.J.; Klein-Nulend, J.

published in
Calcified Tissue International
2011

DOI (link to publisher)
10.1007/s00223-011-9521-1

document version
Publisher's PDF, also known as Version of record

Calcif Tissue Int (2011) 89:318–326
DOI 10.1007/s00223-011-9521-1

ORIGINAL RESEARCH

Mechanical Loading Stimulates BMP7, But Not BMP2, Production by Osteocytes

Ana Santos · Astrid D. Bakker · Hubertine M. E. Willems · Nathalie Bravenboer · Antonius L. J. J. Bronckers · Jenneke Klein-Nulend

Received: 17 May 2011 / Accepted: 22 July 2011 / Published online: 13 August 2011

Abstract Bone mechanical adaptation is a cellular process that allows bones to adapt their mass and structure to mechanical loading. This process is governed by the osteocytes, which in response to mechanical loading produce signaling molecules that affect osteoblasts and osteoclasts. Bone morphogenic proteins (BMPs) are excellent candidates as signaling molecules, but it is unknown whether mechanically stimulated osteocytes affect bone adaptation through BMP production. Therefore, the aim of this study was to assess whether osteocytes produce BMPs in response to mechanical loading. In addition, since BMP7 has a vitamin D receptor (VDR) response element in the promoter region, we also investigated whether VDR is involved in the BMP7 response to mechanical loading. Human or $VDR^{-/-}$ mouse primary bone cells were submitted in vitro to 1 h pulsating fluid flow (PFF) and postincubated without PFF (PI) for 1–24 h, and gene and protein expression of BMP2 and BMP7 were quantified. In human bone cells, PFF did not change BMP2 gene expression, but it upregulated BMP7 gene expression by 4.4- to 5.6-fold at 1–3 h PI and stimulated BMP7 protein expression by 2.4-fold at 6 h PI. PFF did not stimulate BMP7 gene expression in $VDR^{-/-}$ mouse bone cells. These results show for the first time that mechanical loading upregulates BMP7, likely via the VDR, but not BMP2, gene and protein expression in osteocytes in vitro. Since BMP7 plays a major role in bone development and remodeling, these data might contribute to a better understanding of the mechanism leading to the mechanical adaptation of bone.

Keywords Bone adaptation · Bone morphogenic protein 2 · Bone morphogenic protein 7 · Osteocyte · Mechanical loading

Mechanical adaptation of bone is a cellular process that allows bones to adapt their mass and structure to their mechanical environment [1, 2]. It is currently believed that this process of adaptation is governed by the osteocytes [3–6]. Osteocytes are terminally differentiated osteoblasts that become embedded deep within the mineralized bone matrix during bone formation. They are regularly distributed throughout the bone matrix and connected to each other by cytoplasmatic protrusions that run through the canaliculi [7]. This way the osteocytes form a unique dendritic network that enables contact not only with the bone surface but also with other cells [7]. When bones are loaded, the resulting deformation causes a flow of interstitial fluid through the lacunocanalicular network [8, 9]. This flow of fluid results in mechanical stimulation of the osteocytes [6, 8, 9]. The mechanically stimulated osteocytes then produce signaling molecules that are potent regulators of the other types of bone cells, i.e., osteoblasts

The authors have stated that they have no conflict of interest.

A. Santos · A. D. Bakker · H. M. E. Willems · A. L. J. J. Bronckers · J. Klein-Nulend (✉)
Department of Oral Cell Biology, Academic Centre for Dentistry Amsterdam (ACTA), University of Amsterdam and VU University Amsterdam, Research Institute MOVE, Gustav Mahlerlaan 3004, 1081 LA Amsterdam, The Netherlands
e-mail: j.kleinnulend@acta.nl

N. Bravenboer
Department of Endocrinology, VU University Medical Center, Research Institute MOVE, Amsterdam, The Netherlands

N. Bravenboer
Department of Clinical Chemistry, VU University Medical Center, Research Institute MOVE, Amsterdam, The Netherlands

and osteoclasts, during bone remodeling [10, 11]. A multitude of interacting signaling pathways are involved in translating the load applied on bone into the production of signaling molecules by osteocytes [12].

Bone morphogenic proteins (BMPs) are excellent candidates as signaling molecules that could be produced in response to mechanical loading. BMPs belong to a family of genetically conserved secreted signaling molecules of the transforming growth factor-β superfamily of polypeptides (for review, see Chen et al. [13]) [14]. BMPs signal through heteromeric type I and type II serine-threonine receptors and activate the intracellular signalling molecules Smad1, Smad5, and Smad8 through serine phosphorylation [13]. Activated Smad proteins form heterodimers with the co-smad, Smad 4, and translocate to the nucleus to mediate the transcription of BMP-dependent target genes [13].

BMPs might have a relevant role in bone mechanotransduction. BMP2/4 is upregulated by mechanical loading through the induction of Indian hedgehog in chondrocytes, and the BMP antagonist noggin inhibits mechanical loading-induced chondrocyte proliferation [15]. This indicates that BMPs are part of the transduction pathway that is activated in response to mechanical loading in chondrocytes [15]. Mechanical loading of the rat achilles tendon upregulates BMP7 gene expression [16]. It upregulates BMP2 gene expression in endothelial cells of the vascular system [17]. This suggests that BMPs might be part of the signaling pathways that are activated upon mechanical stimulation. Remarkably, the data available on the role of BMPs in bone mechanotransduction are extremely limited.

Systematic administration of BMP7 enhances endosteal bone formation in response to mechanical loading in a rat tibial bending model, suggesting a possible role of BMP7 in the response of bone to mechanical loading in vivo [18]. A loss-of-function mutation at the BMP7 locus resulted in BMP7 knockout mice with specific skeletal defects. The most prevalent effect was the failure of one or both of the seventh-pair ribs to fuse with the sternum. These results suggest that although BMP7 is not limited to skeletal development, BMP7 is involved in bone formation and patterning [19, 20]. Furthermore, primary osteoblasts derived from C57BL/6J mice show upregulation of BMP-related genes, i.e., BMP4, BMP receptor 1, and BMP receptor 2, after mechanical loading by fluid shear stress [12]. Moreover, treatment with noggin ceases the fluid flow-induced osteoblast proliferation [12], indicating that the BMP signaling pathway likely contributes to the translation of fluid shear stress into the anabolic response of bone. However, thus far it is unknown whether mechanical adaptation of bone occurs through BMPs produced by mechanically stimulated osteocytes. To assess whether BMPs play a role in the process of mechanotransduction in bone, we studied the effect of mechanical loading on BMP production by osteocytes, which are considered the prime bone mechanosensor cells [1, 5, 6]. We focused on BMP2 and BMP7 since these BMPs are currently available as clinically approved recombinant human proteins for bone healing [21, 22]. In addition, since BMP7 has a vitamin D receptor (VDR) response element (VDRE) [23], we also investigated whether VDR is involved in the BMP7 response to mechanical loading.

Materials and Methods

Immunohistochemical Analysis

Four 12-week-old female Wistar rats (235 $\pm$ 12 g; Harlan, Zeist, the Netherlands) were killed for immunohistochemical analysis. The animal experiment was performed in accordance with the governmental guidelines for care and use of laboratory animals and approved by the Institutional Animal Care and Use Committee of the VU University Medical Center (Amsterdam, the Netherlands). Tibiae from rats were retrieved and fixed with 4% (w/v) formaldehyde (buffered in phosphate-buffered saline [PBS], pH 7.4) at 4°C for 24 hours. After fixation, the tibiae were decalcified in 10% EDTA containing 0.5% formaldehyde in PBS at 4°C for 4.5 weeks. Tibiae were washed in PBS, dehydrated through an ascending series of ethanol and xylene at room temperature, and embedded in paraffin. After deparaffination in xylene and rehydration in a descending series of ethanol, tissue sections were rinsed in PBS. Endogenous peroxidase was blocked by incubation in 3% H_2O_2 in water for 3 min at room temperature. Potential endogenous binding sites for avidin and biotin were blocked using a biotin avidin blocking kit (Vector Labs, Burlingame, CA) according to the manufacturer's instructions. Nonspecific binding sites were blocked by incubating tissue sections with normal goat serum (30%) for 30 min. Then, sections were incubated overnight at 4°C with primary antibodies to BMP2 [24] (1:50) and BMP7 [25] (1:100), kindly donated by Dr. J. Maliakal. Recombinant hBMP2 (COP-16, cloned in *Escherichia coli*) was used to raise the rabbit anti-BMP2 (rabbit 88) and recombinant hBMP7 (mature form, cloned in Chinese hamster ovary cells) to raise chicken anti-BMP7 (chicken 881). After rinsing in PBS, tissue sections were incubated with biotinylated goat anti-rabbit IgG or biotinylated goat anti-chicken IgG (ABC peroxidase technique, Vectastain, Elite kit; Vector Labs) for 1 h at room temperature, rinsed, and incubated with ABC–peroxidase complex for 1 h at room temperature. After rinsing, the bound peroxidase complex was developed for 6 min at room temperature with DAB kit (Vector Labs) and sections were counterstained with hematoxylin. Kidney sections were used as a positive control. As a negative control,

primary antibodies were replaced by normal rabbit or chicken IgG (Vector Labs).

Primary Human Bone Cells and Primary Mouse Bone Cells

For primary human bone cell isolation, trabecular bone samples (surgical waste) from seven female and two male donors (mean age 43, SD 23.7, range 18–84 years) were obtained from the proximal femoral shaft during hip replacement surgery for coxarthrosis. The protocol was approved by the Ethical Review Board of the VU University Medical Center, and all subjects gave informed consent.

Mouse long bone cells were obtained from the limbs of adult VDR knockout ($VDR^{-/-}$) mice [26]. To isolate $VDR^{-/-}$ mouse bone cells, $VDR^{-/-}$ mice were generated as described previously [26] (kindly provided by Dr. G. Carmeliet, Katholieke Universiteit, Leuven, Belgium). All mice were bred at the local animal housing facility (Proefdierencentrum, Leuven, Belgium) and lived in conventional conditions: 12-h light/dark cycle, standard diet (1% calcium, 0.76% phosphate), and water ad libitum. All experimental procedures were conducted after obtaining formal approval from the ethical committee of the Katholieke Universiteit Leuven.

Human trabecular bone biopsies were placed in sterile PBS, chopped into small fragments, and washed extensively with PBS. Long bones from $VDR^{-/-}$ mice were aseptically harvested, epiphyses were cut off, and bone marrow was flushed out using a syringe and needle. The diaphyses were chopped into small fragments and washed extensively with PBS. Human and mouse bone fragments were treated in exactly the same way, as described previously [27]. To obtain outgrowth of the bone cells, bone fragments were cultured in DMEM (GIBCO, Grand Island, NY) supplemented with 100 U/ml penicillin (Sigma, St. Louis, MO), 50 μg/ml streptomycin sulfate (GIBCO), 50 μg/ml gentamicin (GIBCO), 1.25 μg/ml fungizone (GIBCO), 100 μg/ml ascorbate (Merck, Darmstadt, Germany), and 10% FBS. After reaching subconfluence, outgrowth bone cells were replated at 5×10^3 cells/75 cm^2 flask until enough cells were obtained. All cells used were from passage 2 or less. For pulsating fluid flow (PFF) experiments outgrowth bone cells from humans or $VDR^{-/-}$ mice were harvested and seeded at a density of 5×10^5 cells per polylysine-coated (50 μg/ml; poly-L-lysine hydrobromide, mol wt 15 to 30×10^4; Sigma) glass slide (5 cm^2) and incubated overnight to promote cell attachment.

Pulsating Fluid Flow

Pulsating fluid flow was generated by pumping 13 ml of culture medium, using a roller pump, through a parallel-plate flow chamber as described previously [5, 8, 28]. Briefly, fluid shear stress of 0.7 ± 0.3 Pa at 5 Hz was induced for 1 h on the monolayer of human and mouse primary bone cells by circulating 13 ml of DMEM containing 10% FBS plus antibiotics. Static control cultures (Co) were kept in a Petri dish under similar conditions as the experimental cultures. After 1 h of PFF or control treatment, human primary bone cells were postincubated without PFF (PI) for 1–24 h in 2.5 ml fresh medium and lysed for total RNA isolation or medium was collected for extracellular BMP2 and BMP7 protein quantification. $VDR^{-/-}$ mouse primary bone cells were lysed directly after 1 hour of PFF for total RNA isolation.

Nitric Oxide

Nitric oxide (NO) was measured as nitrite (NO_2^-) accumulation in the medium using Griess reagent containing 1% sulfanylamide, 0.1% naphthylethelene-diamine-dihydrochloride, and 2.5 M H_3PO_4. Serial dilutions of $NaNO_2$ in nonconditioned medium were used as a standard curve. Absorbance was measured at 540 nm with a microplate reader (Bio-Rad Laboratories, Richmond, CA).

Total DNA Content

Human primary bone cells were lysed with 0.5 ml of ice-cold Milli-Q water, harvested on ice, sonicated for 10 min, and centrifuged for 10 min at 2,000 rpm at room temperature. The supernatants were analyzed for total DNA content, which was quantified using the Cyquant Cell Proliferation Assay (Molecular Probes, Eugene, OR) according to the manufacturer's protocol.

Analysis of Gene Expression

Gene expression of rat and human BMP2 and BMP7 was analyzed using Taq-Man® Gene Expression Assays (Applied Biosystems, Foster City, CA) in an ABI Prism 7700 DNA sequence detector. Gene-expression values were normalized for human or mouse GAPDH (Inventoried TaqMan, Gene Expression Assays).

BMP2 and BMP7 Protein Quantification

Immediately after PFF treatment or static control culture, human primary bone cells were postincubated without PFF for 6 or 24 h in 1.5 ml fresh culture medium. At 6 and 24 h, human BMP2 was quantified in the culture medium using the Quantikine® BMP2 Assay (R&D Systems, Minneapolis, MN), and human BMP7 was quantified using the Quantikine BMP7 Assay (R&D Systems) according to the manufacturer's protocol.

Statistical Analysis

For statistical analysis of total DNA content and NO data, differences between control and PFF groups were tested with Student's two-tailed *t* test for paired groups. NO data were log-transformed in order to obtain normality. For statistical analysis of PFF treatment or mechanical loading-over-static control ratios (PFF/Co), human BMP2 and BMP7 gene expression values are expressed as mean ± SEM. Differences of PFF/Co ratios were tested with a one-tailed *t*-test for single group mean and compared to 1 (PFF/control or loading/control = 1, no difference). Values of the total amount of human BMP2 and BMP7 protein are provided as mean ± SEM. Differences between control and PFF groups were tested with *t* test for paired comparison of means. Differences were considered significant if $P < 0.05$.

Results

To determine whether osteocytes express BMP2 in vivo, immunohistochemical staining was performed in rat tibia (Fig. 1). BMP2 staining was intense in chondrocytes of the resting zone and hypertrophic chondrocytes of the growth plate (Fig. 1a). BMP2-positive cells were found in bone marrow (Fig. 1b) and trabecular and cortical bone (Fig. 1b). In cortical bone, osteoblasts stained positive for BMP2 (Fig. 1b, c), while osteocytes stained either clearly positive or completely negative (Fig. 1b, c). No staining was found in negative controls, in which nonimmune IgG was replaced by the primary antibody (Fig. 1d).

To assess whether osteocytes express BMP7 in vivo, immunohistochemical staining against BMP7 was performed in rat tibia (Fig. 2). BMP7 staining was intense in chondrocytes of the resting zone, proliferative zone, and hypertrophic chondrocytes of the growth plate (Fig. 2a). BMP7-positive cells were found in bone marrow (Fig. 2b) and cortical bone (Fig. 2b). In cortical bone osteoblasts stained positive for BMP7 (Fig. 2c). In osteocytes of cortical bone, a gradient of staining intensity in BMP7 was observed from the endosteal to the periosteal side, with osteocytes showing the highest staining intensity on the endosteal side (Fig. 2b, c). No staining was found in negative controls, in which nonimmune IgG replaced the primary antibody (Fig. 2d).

To determine whether osteocytes respond to mechanical loading with modulation of BMP2 and BMP7 gene expression, human primary bone cells were submitted to 1 h of PFF (Fig. 3). No cells were removed by the fluid flow treatment, as assessed by quantification of the DNA content (control 33.4 ± 3.8 ng/ml, PFF 32.5 ± 3.6 ng/ml; $P = 0.5$). NO release was measured after 5 min of PFF as a parameter for bone cell responsiveness. Five minutes of PFF treatment rapidly increased NO production by 3.2-fold ($P = 0.036$, Fig. 3a). One hour PFF increased BMP2 gene expression by 1.3-fold, but this value did not reach significance ($P = 0.2$, Fig. 3b). Although BMP2 gene expression levels decreased after 1 h of PFF treatment followed by 1–3 h of postincubation without PFF (PI), the values did not reach significance ($P = 0.1$ and $P = 0.2$, Fig. 3b). In contrast, 1 h PFF followed by 1–3 h of PI upregulated BMP7 gene expression by 4.4- to 5.6-fold (Fig. 3c), indicating that mechanical loading upregulates BMP7 gene expression in human primary bone cells.

To verify whether PFF-induced changes in gene expression result in increased protein expression, human primary bone cells were submitted to 1 h PFF and postincubated for 6–24 h without PFF (Fig. 4). Under static control conditions, human bone cells produce 144.6 ± 1.6 pM BMP2 protein (Fig. 4a), while basal levels of BMP7 protein are 12.2 ± 1.1 pM (Fig. 4b). This indicates that human primary bone cells produce 11.8-fold more BMP2 than BMP7 ($P = 0.001$) under static control conditions. One hour PFF followed by 6–24 h of PI did not change BMP2 protein levels when compared to static controls (Fig. 4a). However, 1 h PFF followed by 6 h PI significantly upregulated BMP7 protein levels by 2.4-fold (Fig. 4b).

To investigate how osteocytes increase BMP7 production in to mechanical loading, $VDR^{-/-}$ mouse primary bone cells were submitted to mechanical loading by PFF in vitro (Fig. 5). NO release was measured after 5 min of PFF as a parameter for bone cell responsiveness. Five minutes of PFF treatment increased NO production by 1.8-fold ($P = 0.023$, Fig. 5a). Our preliminary data on BMP7 gene expression show that PFF did not change BMP7 gene expression in these cells (Fig. 5b), suggesting that the VDR mediates mechanical loading-induced upregulation of BMP7 in osteocytes.

Discussion

Bone is a living tissue that is able to adapt its mass and structure to mechanical demands throughout life [2]. Osteocytes are proposed as the key cells that convert the physical stimuli resulting from daily loading into a biological signal by the secretion of signaling molecules in a process known as mechanotransduction [1, 5, 8]. These mechanostimulated molecules activate a wide spectrum of signaling pathways fundamental for bone resorption by osteoclasts and/or bone formation by osteoblasts [12]. BMPs represent a class of key proteins for the regulation of cell–cell communication, cell differentiation, and morphogenesis of several organs [13, 29]. In bone, BMPs are

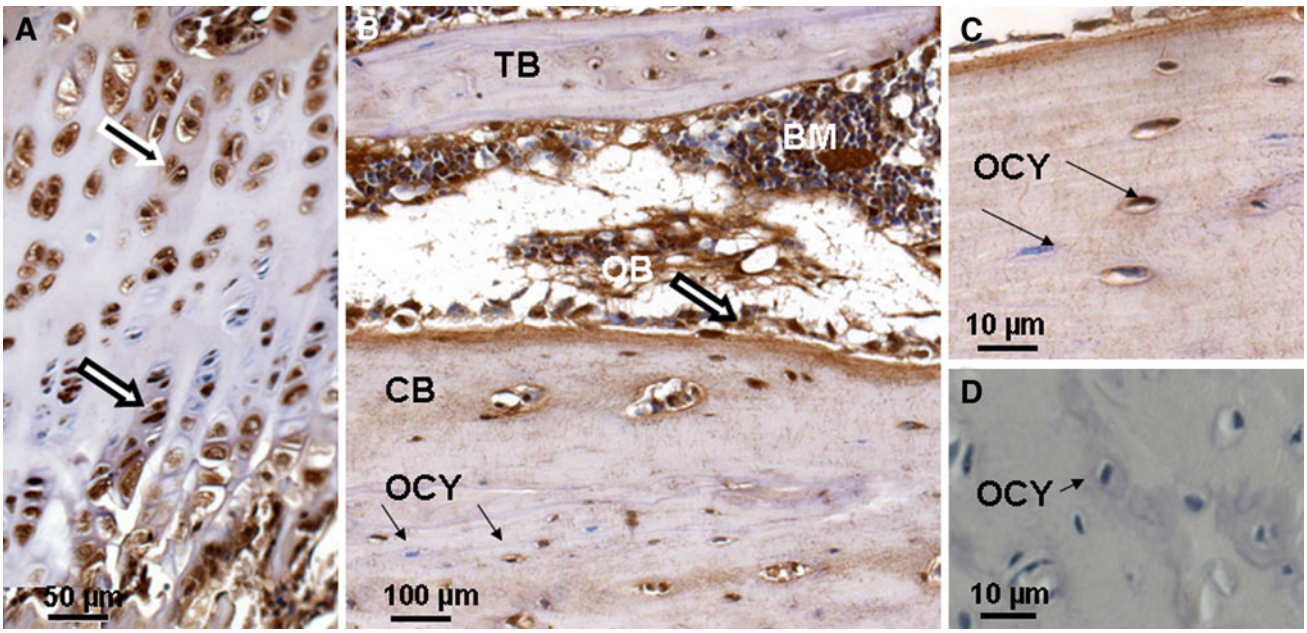

Fig. 1 BMP2 expression in rat tibia. **a** BMP2-positive staining (*brown*) was intense in chondrocytes of the resting zone (*white arrow*) and in hypertrophic chondrocytes of the growth plate (*black arrow*). **b** BMP2-positive cells were found in bone marrow (*BM*), trabecular bone (*TB*), and cortical bone (*CB*). In cortical bone, osteoblasts (*OB*, *white arrow*) stained positive for BMP2, while osteocytes (*OCY*, *black arrows*) stained either clearly positive or completely negative. **c** High magnification of cortical bone showing osteocytes that stained positive or negative for BMP2. **d** Negative control

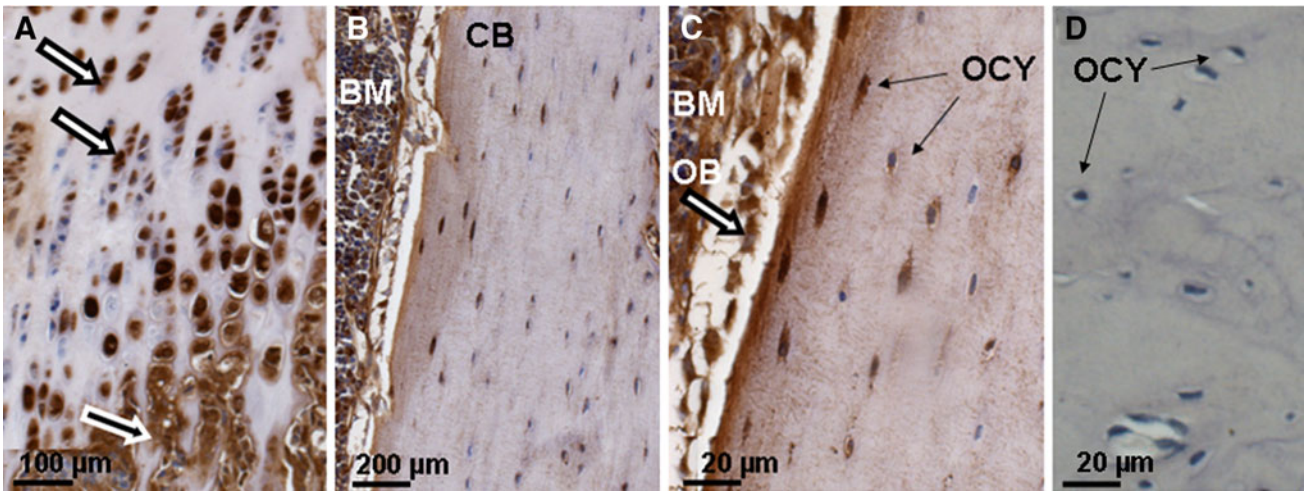

Fig. 2 BMP7 expression in rat tibia. **a** BMP7-positive staining (*brown*) was intense in chondrocytes of the resting zone (*white arrow*) and in hypertrophic chondrocytes of the growth plate (*black arrow*). **b** BMP7-positive cells were found in bone marrow (*BM*) and cortical bone (*CB*). **c** High magnification of cortical bone. **d** Negative control. In cortical bone, a gradient staining intensity of BMP7-positive cells was observed, with osteoblasts (*OB*, *white arrow*) and osteocytes (*OCY*, *black arrows*) at the endosteal surface mostly staining positive for BMP7

multifunctional regulators of proliferation and differentiation during development [30, 31] and are required for osteoblast gene expression and differentiation [30–33]. BMPs represent one of the most studied growth factors due to their relevance in bone regeneration. However, it is unclear what role BMPs, specifically BMP2 and BMP7, play in the process of bone mechanotransduction. Therefore, we studied the effect of mechanical loading on BMP production by osteocytes, which are considered the prime bone mechanosensor cells [1, 3–5].

We used human primary bone cells obtained as outgrowth from collagenase-stripped bone pieces to study

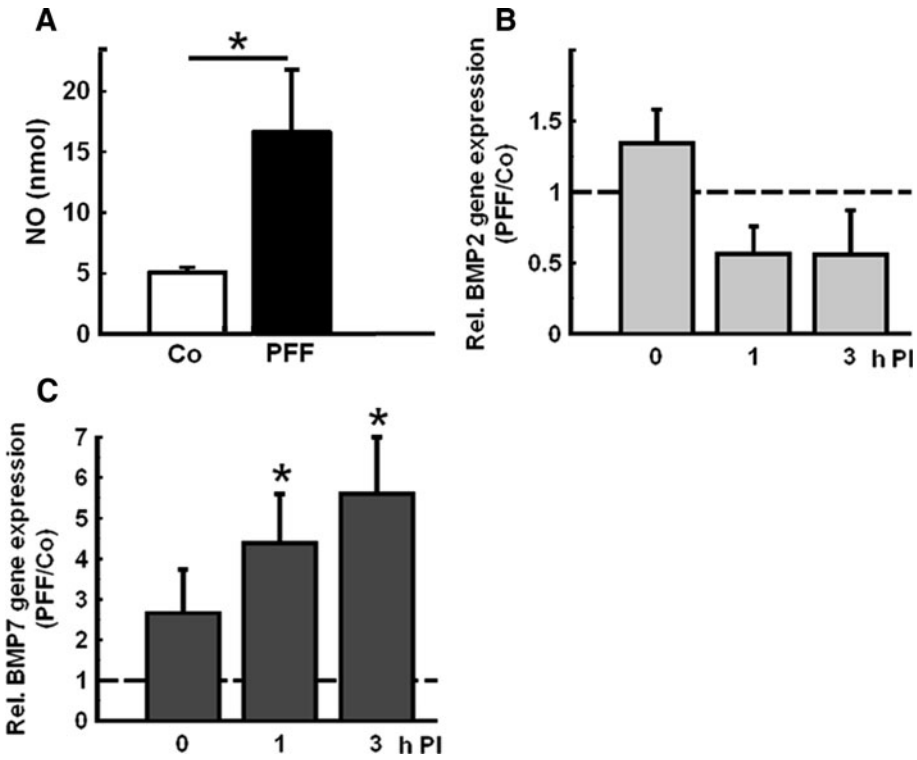

Fig. 3 PFF increases NO production and upregulates gene expression of BMP7, but not BMP2, in human primary bone cells. **a** Five minutes of mechanical loading by PFF enhanced NO production. **b** PFF did not affect BMP2 gene expression. **c** One hour of PFF followed by 1 and 3 hours of PI upregulated BMP7 gene expression. Values were normalized for human GAPDH and expressed as mean ± SEM of absolute values and PFF-over-control ratios of five independent donors. *PFF* pulsating fluid flow, *Co* static control, *PI* postincubation without PFF. *Significant effect of PFF, $P < 0.05$

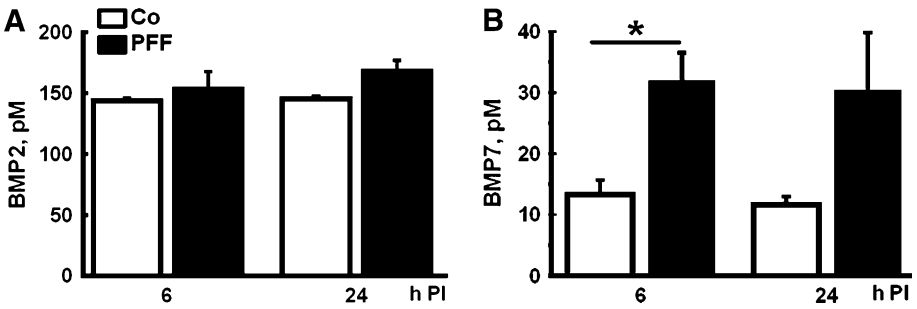

Fig. 4 PFF upregulates protein expression of BMP7, but not BMP2, in human primary bone cells. **a** PFF did not affect protein expression of BMP2. **b** One hour of PFF followed by 6 and 3 hours of PI upregulated BMP7 protein expression. Values were expressed as mean ± SEM of PFF-over-static control ratios of five independent donors. PFF, pulsating fluid flow; Co, static control; PI, postincubation without PFF. *Significant effect of PFF, $P < 0.05$

whether osteocytes in vitro respond to mechanical loading with production of BMP2 and BMP7. Although the exact nature of the outgrowth cells is not completely known, human primary bone cells may be considered bone cells since they express alkaline phosphatase, cfba-I [32, 33], and osteocalcin [32–34]. In addition, these cells respond to mechanical loading with increased expression of COX-2 [23], indicating that these bone cell cultures have an osteocyte-like phenotype since mechanical loading induces increased gene expression of COX-2 in chicken osteocytes but not osteoblasts [35]. Furthermore, these cells express detectable levels of MEPE, DMP1, and SOST (unpublished observations), which are considered osteocyte markers [36].

We found that PFF, representing a physiological loading regime [28], significantly increased BMP7, but not BMP2, gene and protein expression in human osteocytes, indicating that upregulation of BMP7 represents a physiological response to mechanical loading of bone. Our findings are supported by the observation of others that load-induced bone formation in vivo is enhanced by BMP7 [18], indicating that BMP7 might be an essential molecule in the process of mechanically driven bone formation.

Contradictory data have been reported on the effects of mechanical loading on BMP production in bone. Compressive forces and shear stress upregulate expression of BMPs 2, 4, 6, and 7 in osteoblasts in vitro [12, 37]. Upregulation of BMPs 2, 4, and 6 was observed during

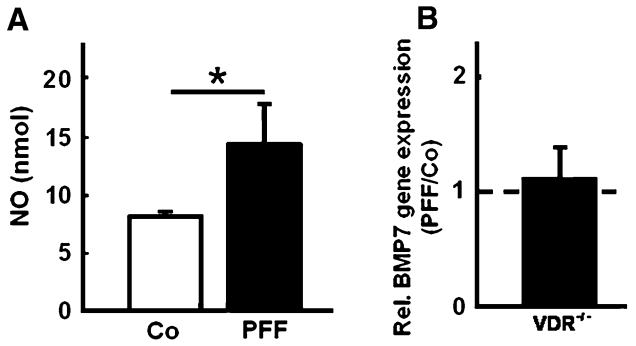

Fig. 5 PFF increases NO production but does not alter gene expression of BMP7 in $VDR^{-/-}$ mouse primary bone cells. **a** 5 min of mechanical loading by PFF enhanced NO production. **b** PFF did not affect gene expression of BMP7 in $VDR^{-/-}$ mouse primary bone cells. Values were normalized for mouse GAPDH and expressed as mean ≤ SEM of absolute values and PFF-over-control ratios of three independent donors. *PFF* pulsating fluid flow, *Co* static control. *Significant effect of PFF, $P < 0.05$

distraction osteogenesis; but BMP7 expression did not change during the distraction [38]. Mechanical tension during distraction osteogenesis in vivo increases BMP2 and BMP4, but not BMP7, gene expression [39]. These observations differ from our results, showing that mechanical loading stimulated BMP7, but not BMP2, expression in cultured osteocytes. In addition, we assessed expression of BMP4 and BMP6 (data not shown). BMP4 gene expression seemed to be reduced after PFF treatment. BMP6 gene-expression data were not consistent; therefore, we cannot exclude a role for BMP6 in the anabolic response of osteocytes to mechanical loading. It is generally accepted that osteocytes are the primary sensors of mechanical loading in bone, rather than osteoblasts [1, 5–8]. Osteocytes and osteoblasts respond differently in terms of loading-induced production of signaling molecules [2, 8, 11]. Therefore, differences in mechanical loading regimes and characteristics between osteocytes and osteoblasts might explain the differences in loading-induced BMP expression between experiments.

To investigate how osteocytes increase BMP7 production in response to mechanical loading, we searched for signaling pathways that are activated upon loading and could mediate this response. Since both BMP7 protein expression and gene expression were affected by loading, we focused on signaling pathways that activate the BMP7 promoter sequence. BMP2 gene expression remained unchanged, but BMP7 gene expression and protein expression were affected by mechanical loading, although BMPs are highly conserved proteins. Thus, we focused on possible differences between the promoter region of BMP2 and BMP7. The BMP7, but not the BMP2, promoter does contain VDREs [23]. Therefore, we hypothesized that the VDR is involved in the increase in BMP7 gene expression in response to mechanical loading. To test our hypothesis, $VDR^{-/-}$ mouse primary bone cells [26] were submitted to mechanical loading by PFF in vitro. Our data show that PFF does not change BMP7 gene expression in these cells (Fig. 5), suggesting that the VDR mediates mechanical loading-induced upregulation of BMP7 in osteocytes. Although $VDR^{-/-}$ mouse primary bone cells were derived from mice rather than humans, they were obtained in exactly the same manner. In addition, mouse and human primary bone cells show the same response to mechanical loading as illustrated by the increased NO production in response to mechanical loading.

It is well established that bone cells and chondrocytes produce BMPs [13]. Similar to observations by others, we also show moderate to intense expression of BMP2 and BMP7 in bone marrow cells, bone lining cells, osteoblasts, and osteocytes [40, 41]. We observed a gradient of BMP7 expression, varying from intense staining at the endosteal side to no staining at the periosteal side of cortical bone. In addition, basal levels of BMP2 expression were significantly higher than basal BMP7 expression in cultured human osteocytes, and BMP7, but not BMP2, was produced in response to mechanical loading. Since BMP2 is essential for recruitment of bone marrow cells and initial cell lineage commitment, while BMP7 has a relevant role in the maturation process of preosteoblasts, these finding might have implications for bone remodeling. However, the role of BMPs in bone remodeling is not fully understood. BMP2 is produced by bone marrow stromal cells and necessary for osteoblast differentiation [40, 41]. Cheng and colleagues [42] reported an osteogenic hierarchy where BMPs 2, 6, and 9 are the most potent agents to induce osteoblast lineage-specific differentiation of mesenchymal progenitor cells, while most BMPs can promote the terminal differentiation of committed osteoblast precursors and osteoblasts. BMP7 increases bone formation primarily by promoting preosteoblast growth and differentiation without affecting bone matrix production by individual osteoblasts in vitro [43]. Systemic administration of BMP7 stimulates the differentiation of bone lining cells, fibroblasts, and other committed cells to osteoblasts but does not increase putative stem cell proliferation [18]. Thus, it is possible that the mature cells of the osteoblastic lineage, i.e., the osteocytes, respond to mechanical loading with increased BMP7 production to enhance differentiation of already committed osteogenic cells, while high basal BMP2 expression guarantees the differentiation of bone marrow cells into preosteoblasts. In other words, BMP2 seems to be essential for general bone formation, while BMP7 is required for local bone formation in response to mechanical loading. Future functional studies are needed to assess the precise functional role of loading-induced BMP7 production in bone (re)modeling.

In bone biology, the mechanisms by which bones adapt to mechanical demands are still a challenging area of research. We show for the first time that physiological loading regimes induce increased BMP7 gene and protein expression in osteocytes, the bone mechanosensor cells. Basal BMP2 protein expression levels were significantly higher than BMP7 levels in osteocytes, while mechanical loading did not affect BMP2 gene or protein expression. Thus, it is likely that BMP7 plays a role in mechanotransduction in osteocytes. Our data might contribute to a better understanding of the mechanisms that lead to bone mechanical adaptation. Unraveling the signaling pathways that lead to an optimal response to physiological loading regimes might provide new insights for therapeutic approaches in bone biology.

Acknowledgments The Dutch Program for Tissue Engineering supported the work of A. S. (DPTE grant V6T6744). The Research Institute MOVE of the VU University Amsterdam supported the work of A. D. B. The work of H. M. E. W. was supported by a grant from Royal FrieslandCampina (Deventer, the Netherlands). The authors thank J. M. A. Hogervorst for excellent help in performing mechanical loading experiments.

References

1. Burger EH, Klein-Nulend J (1999) Mechanotransduction in bone—role of the lacuno-canalicular network. FASEB J 13 Suppl:S101–S112
2. Frost HM (1987) Bone "mass" and the mechanostat": a proposal. Anat Rec 219:1–9
3. Aarden EM, Burger EH, Nijweide PJ (1994) Function of osteocytes in bone. J Cell Biochem 55:287–299
4. Tatsumi S, Ishii K, Amizuka N, Li M, Kobayashi T, Kohno K, Ito M, Takeshita S, Ikeda K (2007) Targeted ablation of osteocytes induces osteoporosis with defective mechanotransduction. Cell Metab 6:464–475
5. Klein-Nulend J, van der Plas A, Semeins CM, Ajubi NE, Frangos JA, Nijweide PJ, Burger EH (1995) Sensitivity of osteocytes to biomechanical stress in vitro. FASEB J 9:441–445
6. Cowin SC, Moss-Salentijn L, Moss ML (1991) Candidates for the mechanosensory system in bone. J Biomech Eng 113:191–197
7. Kamioka H, Honjo T, Takano-Yamamoto T (2001) A three dimensional distribution of osteocyte processes revealed by combination of confocal laser microscopy and differential interference contrast microscopy. Bone 28:145–149
8. Klein-Nulend J, Semeins CM, Ajubi NE, Nijweide PJ, Burger EH (1995) Pulsating fluid flow increases nitric oxide (NO) synthesis by osteocytes but not periosteal fibroblasts—correlation with prostaglandin upregulation. Biochem Biophys Res Commun 217:640–648
9. Ciani C, Doty SB, Fritton SP (2009) An effective histological staining process to visualize bone interstitial fluid space using confocal microscopy. Bone 44:1015–1017
10. Tan SD, de Vries TJ, Kuijpers-Jagtman AM, Semeins CM, Everts V, Klein-Nulend J (2007) Osteocytes subjected to fluid flow inhibit osteoclast formation and bone resorption. Bone 41:745–751
11. Vezeridis PS, Semeins CM, Chen Q, Klein-Nulend J (2006) Osteocytes subjected to pulsating fluid flow regulate osteoblast proliferation and differentiation. Biochem Biophys Res Commun 348:1082–1088
12. Lau KH, Kapur S, Kesavan C, Baylink DJ (2006) Upregulation of the Wnt, estrogen receptor, insulin-like growth factor-I, and bone morphogenetic protein pathways in C57BL/6J osteoblasts as opposed to C3H/HeJ osteoblasts in part contributes to the differential anabolic response to fluid shear. J Biol Chem 281:9576–9588
13. Chen D, Zhao M, Mundy GR (2004) Bone morphogenic proteins. Growth Factors 22:233–241
14. Miyazono K, Kamiya Y, Morikawa M (2010) Bone morphogenetic protein receptors and signal transduction. J Biochem 147:35–51
15. Wu Q, Zhang Y, Chen Q (2001) Indian Hedgehog is an essential component of mechanotransduction complex to stimulate chondrocyte proliferation. J Biol Chem 276:3529–3596
16. Eliasson P, Fahgren A, Aspenberg P (2008) Mechanical load and BMP signaling during tendon repair: a role for follistatin? Clin Orthop Relat Res 466:1528–1532
17. Czisar A, Smith KE, Koller A, Kaley G, Edwards JG, Ungvari Z (2005) Regulation of bone morphogenic protein 2 expression in endothelial cells. Role of the nuclear factor-κB activation by tumor necrosis factor-α, H_2O_2, and high intravascular pressure. Circulation 111:2364–2372
18. Cheline AJ, Reddi AH, Martin RB (2002) Bone morphogenic protein-7 selectively enhances mechanically induced bone formation. Bone 31:570–574
19. Dudley AT, Lyons KM, Robertson EJ (1995) A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. Genes Dev 9:2795–2807
20. Luo G, Hofmann C, Bronckers AL, Sohocki M, Bradley A, Karsenty G (1995) BMP-7 is an inducer of nephrogenesis, and is also required for eye development and skeletal patterning. Genes Dev 9:2808–2820
21. Sampath TK, Maliakal JC, Haushka PV, Jones WK, Sasal H, Tucker RF, White KH, Coughlin JE, Tucker MM, Pang RH (1992) Recombinant human osteogenic protein-1 (hOP-1) induces new bone formation in vivo with a specific activity comparable with natural bovine osteogenic protein and stimulates osteoblast proliferation and differentiation in vitro. J Biol Chem 20:20352–20362
22. Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Kriz RW, Hewik RM, Wang EA (1998) Novel regulators of bone formation: molecular clones and activities. Science 242:1528–1534
23. Pálmer HG, Anjos-Afonso F, Carmeliet G, Takeda H, Watt FM (2008) The vitamin D receptor is a Wnt effector that controls hair follicle differentiation and specifies tumor type in adult epidermis. PLoS One 23:e1483
24. Zoricic S, Maric I, Bobinac D, Vukicevic S (2003) Expression of bone morphogenetic proteins and cartilage-derived morphogenetic proteins during osteophyte formation in humans. J Anat 202:269–277
25. Helder MN, Karg H, Bervoets TJ, Vukicevic S, Burger EH, D'Souza RN, Wöltgens JH, Karsenty G, Bronckers AL (1998) Bone morphogenetic protein-7 (osteogenic protein-1, OP-1) and tooth development. J Dent Res 77:545–554
26. Van Cromphaut SJ, Dewerchin M, Hoenderop JG, Stockmans I, Van Herck E, Kato S, Bindels RJ, Collen D, Carmeliet P, Bouillon R, Carmeliet G (2001) Duodenal calcium absorption in vitamin D receptor-knockout mice: functional and molecular aspects. Proc Natl Acad Sci USA 98:13324–13329
27. Bakker AD, Klein-Nulend J, Burger EH (2003) Mechanotransduction in bone cells proceeds via activation of COX-2, but not COX-1. Biochem Biophys Res Commun 305:677–683
28. Bacabac RG, Smit TH, Cowin SC, Van Loon JJWA, Nieuwstad FT, Heethaar R, Klein-Nulend J (2005) Dynamic shear stress in parallel-plate flow chambers. J Biomech 38:159–167

29. Zhao GQ (2003) Consequences of knocking out BMP signalling in the mouse. Genesis 35:43–56
30. Zhang H, Bradley A (1996) Mice deficient for BMP2 are nonviable and have defects on the amnion/chorion and cardiac development. Development 122:2977–2986
31. Mishina Y, Suzuki A, Ueno N, Behringer RR (1995) Bmpr encodes type I bone morphogenic protein receptor that is essential for gastrulation during mouse embryogenesis. Genes Dev 9:3027–3037
32. Luu HH, Song WX, Luo X, Manning D, Luo J, Deng Z, Sharff KA, Montag AG, Haydon RC, He TC (2007) Distinct roles of bone morphogenic proteins in osteogenic differentiation of mesenchymal stem cells. J Orthop Res 25:665–677
33. Gronthos S, Zannettino ACW, Graves SE, Otha S, Hay SJ, Simmons PJ (1999) Differential cell surface expression of the stro-I and alkaline phosphatase antigens on discrete developmental stages in primary cultures of human bone cells. J Bone Miner Res 14:47–56
34. Sterck JG, Klein-Nulend J, Lips P, Burger EH (1998) Response of normal and osteoporotic human bone cells to mechanical stress in vitro. Am J Physiol Endocrinol Metab 274:E113–E120
35. Westbroek I, Ajubi NE, Alblas MJ, Semeins CM, Klein-Nulend J, Burger EH, Nijweide PJ (2000) Differential stimulation of prostaglandin G/H synthase-2 in osteocytes and other osteogenic cells by pulsating fluid flow. Biochem Biophys Res Commun 268:414–419
36. Bonewald LF, Johnson ML (2008) Osteocytes, mechanosensing and Wnt signaling. Bone 42:606–615
37. Mistui N, Suzuki N, Maeno M, Yanagisawa M, Koyama Y, Otsuka K, Shimizu N (2006) Optimal compressive forces induce bone formation via increasing bone morphogenic proteins production and decreasing their antagonists production by Saos-2 cells. Life Sci 78:2697–2706
38. Haque T, Hamade F, Alam N, Kotsiopriftis M, Lauzier D, St-Arnaud R, Hamdy RC (2008) Characterizing the BMP pathway in a wild type mouse model of distraction osteogenesis. Bone 42:1144–1153
39. Sato M, Ochi T, Nakase T, Hirota S, Kitamura Y, Nomura S, Yasu N (1999) Mechanical tension-stress induces expression of bone morphogenic protein (BMP-2) and BMP-4, but not BMP-6, BMP-7, and GDF-5 mRNA, during distraction osteogenesis. J Bone Miner Res 14:1084–1095
40. McCullough KA, Waits CA, Garimella R, Tague S, Sipe JB, Anderson HC (2007) Immunohistochemical localization of bone morphogenic proteins (BMPs) 2, 4, 6, and 7 during induced heterotopic bone formation. J Orthop Res 25:465–472
41. Abe E, Yammamoto M, Taguchi Y, Lecka-Czernik B, O'Brien CA, Economides AN, Stahl N, jilka RL, Manologas SC (2000) Essential requirement of BMP2/4 for both osteoblast and osteoclast formation in murine bone marrow cultures from adult mice: antagonism by Noggin. J Bone Miner Res 15:663–673
42. Cheng H, Jiang W, Phillips FM, Haydon RC, Peng Y, Zhou L, Luu HH, An N, Breyer B, Vanischakarn P, Szatkowski JP, Park JY, He TC (2003) Osteogenic activity of fourteen types of human bone morphogenic proteins (BMPs). J Bone Joint Surg Am 85:1544–1552
43. Li IWS, Cheifetz S, McCulloch CAG, Sampath KT, Sodek J (1996) Effects of osteogenic protein-1 (OP-1) on bone matrix protein expression by fetal calvarial cells are differentiation stage specific. J Cell Physiol 169:115–125